

# Expression of the thymidylate synthetase gene of the *Bacillus subtilis* bacteriophage Phi-3-T in *Escherichia coli*

(DNA cloning/plasmid/transformation/hybridization)

S. D. EHRLICH, H. BURSZTYN-PETTEGREW, I. STROYNOWSKI, AND J. LEDERBERG

Department of Genetics, Stanford University Medical School, Stanford, California 94305

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**ABSTRACT** The thymidylate synthetase gene of *B. subtilis* bacteriophage Phi-3-T, when cloned in plasmids pSC101 or pMB9, is expressed in *E. coli*. The promoter of the cloned gene is likely to originate in Phi-3-T. Rearrangements of hybrid plasmid sequences during the cloning have been noted. *B. subtilis* strains can be transformed with hybrid DNAs. The transformants contain sequences of Phi-3-T, but not those of plasmid vectors.

Phi-3-T is a *Bacillus subtilis* temperate bacteriophage capable of lysogenic conversion of *thy*<sup>-</sup> *B. subtilis* clones to prototrophy (1); we describe here the cloning of the synthetase gene carried by Phi-3-T on the *Escherichia coli* plasmids pSC101 (2) and pMB9. The gene complements thymine-deficiency mutation in *E. coli* which indicates its correct transcription and translation in this new host. The promoter of the gene, cloned in pSC101, is likely to be contained within the inserted segment. Hybrid plasmid DNA transforms *B. subtilis*, although about 100-fold less efficiently than the intact phage DNA. *B. subtilis* clones transformed with the hybrid plasmid, DNA do not contain detectable pSC101 sequences, but do show sequences homologous with part of the Phi-3-T genome.

## MATERIALS AND METHODS

**Bacterial Strains.** *E. coli* strains used were W5443 *thr-1 leu-6 thi-1 supE44 lacY1 r<sup>-</sup> m<sup>-</sup> thy<sup>-</sup> str<sup>r</sup> (tonB tryp<sup>-</sup> delta)* (SB2 of D. Finnegan), W5469 *thy<sup>-</sup> his<sup>-</sup> argA metB leu<sup>-</sup> xyl<sup>-</sup> lacY strA polA<sub>18214</sub>* from D. Helinski, C600(pSC101) from S. Cohen, HB129 (pMB9) *endoI<sup>-</sup> r<sub>B</sub><sup>+</sup> m<sub>B</sub><sup>+</sup> gal<sup>-</sup> lac<sup>-</sup> strep<sup>r</sup> leu<sup>-</sup> pro<sup>-</sup> thi<sup>-</sup>* from H. Boyer, and C600 (ColEIamp) (3) from V. Hershfield. *B. subtilis* strains included SB168 *trypC2*, SB591 *thy<sup>-</sup>*, SB1158 *thyA (thyB ilvD6 delta)* from this laboratory and SB168(Phi-3-T) from D. Dean.

**DNAs and Enzymes.** Phage, purified by differential centrifugation followed by two CsCl density bandings, was used to prepare Phi-3-T DNA by phenol extraction. Plasmid DNAs were prepared by the clear lysis procedure (4).

*EcoRI* nuclease was prepared according to an unpublished procedure (T. Landers, personal communication). T4 ligase was prepared and used as described (5). *EcoRI* cleavage was done as described (6). RNA polymerase was a gift of D. Brutlag. <sup>32</sup>P-Labeled complementary RNA [cRNA (1)] was synthesized on DNA templates with RNA polymerase as described (7).

**Transformation Procedures.** Competence induction and transformation of *E. coli* and *B. subtilis* strains was done according to published procedures (8, 9). Cautions appropriate for a P<sub>2</sub> level of containment were adopted for the relevant parts of this investigation.

**Electrophoresis and Electron Microscopy.** Separations were

done on horizontal agarose slab gels essentially as described (6). Heteroduplex analysis was done according to ref 10.

**Hybridization.** *E. coli* and *B. subtilis* colonies on Millipore filters were prepared for *in situ* hybridization according to unpublished procedure of J. Lis and L. Prestidge (personal communication). Hybridization of cRNA treated clones or to DNA segments after electrophoretic separation was as described (11, 12).

**Removal of pSC101-thy Hybrid Plasmid from *E. coli*.** About 1000 cells were inoculated per ml of L-broth containing 120 µg/ml of ethidium bromide and grown overnight. A 0.1 ml aliquot of the culture was diluted into 5 ml of L-broth. After 1 hr at 37°, 20 µg/ml of tetracycline was added, and after another hour 50 µg/ml of ampicillin was added. Two hours later, cells were spun down, resuspended in L-broth, and plated on rich medium. Colonies appearing overnight were replica plated on medium containing tetracycline: one out of eight experiments yielded Tc<sup>s</sup> clones free of plasmid.

## RESULTS

### *EcoRI* segments of Phi-3-T DNA

The *EcoRI* cleavage pattern of the Phi-3-T DNA observed in the present work agrees with that reported by Wilson *et al.* (13). We could resolve about 30 segments; their sizes, relative to *EcoRI*-cleaved SPPI DNA (14), are displayed in Table 1. The sum of their molecular weights is close to 72 million; this is somewhat lower than the 82.6 ± 5.3 million measured by electron microscopy with the lambda or pSC101 DNA as a standard. The discrepancy probably results from one or more of the following factors: repetition within the phage genome (see below), incomplete resolution by the gel electrophoresis, and the use of different molecular weight standards for the two measurements.

### Isolation of Tc<sup>r</sup> thy<sup>+</sup> *E. coli* transformants

Cleavage of the Phi-3-T DNA with *EcoRI* enzyme decreases the transforming activity of the *thy* gene about 1000 times (not shown). We have, therefore, attempted the cloning of that gene from an incompletely digested DNA sample, in which the transforming activity has been decreased only 10-fold. The sample was ligated to *EcoRI* cleaved pSC101 DNA and used to transform thymine-requiring *E. coli* cells by selecting either for thymine independence or tetracycline resistance. About 400 Tc<sup>r</sup> clones were inspected for the presence of hybrid plasmids by colony hybridization (11). Nearly 8% of the clones contained sequences complementary to Phi-3-T cRNA. Two of the Tc<sup>r</sup> clones showed a *thy*<sup>+</sup> phenotype. Two more *thy*<sup>+</sup> clones have been obtained by selecting that phenotype directly: these were also Tc<sup>r</sup>. The frequency of phenotype occurrence for the di-

Abbreviation: pFT, a cloned plasmid chosen on the basis of its ability to hybridize with Phi-3-T cRNA.

Table 1. Molecular weight of Phi-3-T DNA segments after *Eco*RI cleavage

Band	Molecular weight × 10 <sup>-6</sup>
1	9.4
2	7.9
3	5.4
4	4.9
5 a,b	4.5, 4.4
6	3.9
7 a,b	3.2, 3.1
8	2.9
9	2.5
10 a,b,c	2.07, 2.05, 2.05
11	1.96
12 a,b	1.70, 1.70
13	1.45
14	1.25
15	1.05
16	0.90
17	0.70
18	0.67
19	0.65
20	0.46
21	0.41
22	0.26
23	0.24
24	0.19

The total molecular weight was 71.86.

rectly selected *thy*<sup>+</sup> character is about 10<sup>-8</sup>, compared to 10<sup>-7</sup> if the Tc<sup>r</sup> is selected first.

**Molecular structure of hybrid plasmids**

Plasmids from bacterial clones that hybridize with Phi-3-T cRNA are denoted as "pFT", followed by the clone number. The *Eco*RI restriction pattern of the four DNAs extracted from the Tc<sup>r</sup>*thy*<sup>+</sup> transformants revealed that, in addition to the pSC101 part of the molecule, they all contain one or several DNA segments. Molecular weights of the added segments, their order, and orientation—as determined by electron microscopic inspection of heteroduplexes between the four hybrid plasmids and electrophoretic analysis after partial *Eco*RI cleavage—are shown in Fig. 1. Common to all of the plasmids are sequences of segment A whose size corresponds to segment 5a of the Phi-3-T. That segment then presumably contains the *thy* gene. Within the segment A of the pFT33 an insert of unknown origin is found, which forms a hairpin with the stem about a hundred nucleotides long, when the pFT33 DNA is denatured and rapidly renatured (Fig. 1E). Segments B and C correspond in size to segments 18 and 23 of the phage. Segment D of the plasmids pFT25 and 33 is not released by the action of *Eco*RI nuclease. It is not homologous to pSC101: therefore, it must have arisen as a consequence of a rearrangement during the cloning procedure, which has led to replacement of a part of pSC101 containing an *Eco*RI site with a Phi-3-T segment (see below)

**The *thy*<sup>+</sup> character is plasmid borne**

Three lines of evidence prove that the *thy*<sup>+</sup> gene is harbored on a plasmid. First, hybrid plasmid DNAs, containing less than 1% of linear molecules, transform Tc<sup>r</sup>*thy*<sup>-</sup> *E. coli* to Tc<sup>r</sup>*thy*<sup>+</sup> phenotype (Fig. 2). Transformants selected either for Tc<sup>r</sup> or for *thy*<sup>+</sup> carry the unselected marker as well. The efficiency of

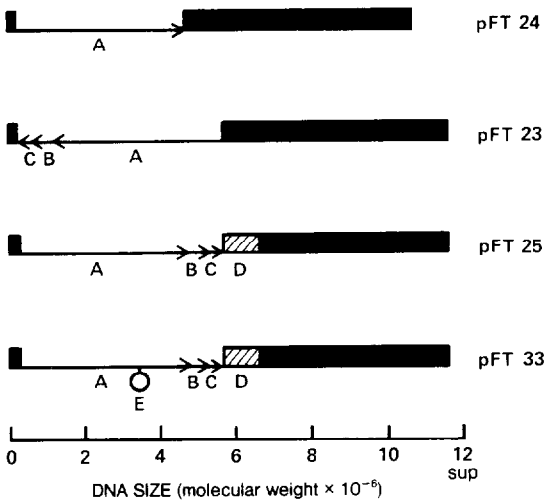


FIG. 1. Molecular structure of four pSC101-*thy* hybrid plasmids. DNAs are aligned from the *Bam* site in pSC101 (0.2 million from the *Eco*RI site, not shown). The pSC101 segment of the hybrid is represented by a heavy line, the insert by a lighter one. There is, in all cases, an *Eco*RI site at the border of pSC101 and the insert. Additional *Eco*RI sites are denoted as arrows. The direction of arrows indicates the orientation of inserts. Part of pSC101 displaced by Phi-3-T sequences (see text) is represented by a hatched thick line. Transposon within the segment A of pFT33 (see text) is represented by a circle. The molecular weight of the segments (in millions) were: A, 4.5; B, 0.67; C, 0.24; D, 0.9; E, 0.9.

transformation, with hybrid plasmid DNAs is the same as that of pSC101 DNA (Fig. 2; Table 2).

Second, *E. coli* transformants of the Tc<sup>r</sup>*thy*<sup>+</sup> phenotype freed of the plasmid (see *Materials and Methods*) lose the Tc<sup>r</sup>*thy*<sup>+</sup> phenotype. Both markers can be reintroduced into the cured strain at a frequency identical to that of the parent, by transformation with the hybrid plasmid DNA.

Third, all the hybrid plasmid DNAs transform thymine requiring *B. subtilis* strains to prototrophy. A representative experiment is shown in Fig. 3, the summary of the efficiencies reported in Table 2. Intact Phi-3-T DNA transforms approximately 100-fold more efficiently than hybrid plasmid DNAs. The low efficiency of the latter is not altered by *Bam* endonuclease, which linearizes the molecule by making a cut in the Tc

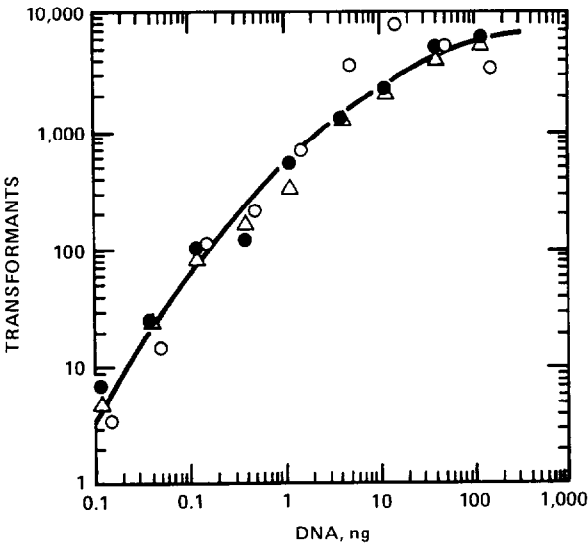


FIG. 2. Transformation of *E. coli* with pSC101 (Tc<sup>r</sup>, open circles) and pFT24 (Tc<sup>r</sup>, triangles; Tc<sup>r</sup>*thy*<sup>+</sup>, closed circles) DNA.

Table 2. Transforming efficiencies of hybrid plasmids

Cells	DNA	Efficiency (cge)*
<i>E. coli</i> W5443	pSC101	10 <sup>-5</sup>
	pFT's	10 <sup>-5</sup>
<i>B. subtilis</i> SB591	φ3T	10 <sup>-4</sup>
	φ3T/ <i>Bam</i>	10 <sup>-5</sup>
	φ3T/RI	10 <sup>-7</sup>
	pFT's	10 <sup>-6</sup>
	pFT's/ <i>Bam</i>	10 <sup>-6</sup>
	pFT's/RI	10 <sup>-7</sup>

\* Colonies per genome equivalent.

gene of the pSC101 component of the hybrid. Similar treatment of the Phi-3-T DNA leads to a 10-fold decrease in transforming activity. The *Eco*RI cleavage reduces the biological efficiency of hybrid plasmids approximately 10-fold while the activity of the phage DNA is impaired 1000-fold. Therefore, the *Eco*RI segment embodying the *thy* gene, displays the same transforming efficiency regardless of which vector carried it (Phi-3-T phage, the hybrid plasmid) or its previous host. These results reaffirm the absence of a restriction system in *B. subtilis* strain SB168 (15), from which our thymine requiring strain is derived.

#### Hybrid plasmids contain Phi-3-T sequences

Several lines of evidence demonstrate the presence of Phi-3-T sequences in the hybrid plasmid DNAs. As already mentioned, the *E. coli* clones harboring these plasmids hybridize with Phi-3-T cRNA. Similarly, *thy*<sup>-</sup> *B. subtilis* strains transformed to thymine independence with pFT DNAs acquire the ability to hybridize Phi-3-T cRNA.

Fig. 4 shows that the colony hybridization technique (11) can be used with *B. subtilis* clones: SB168, a standard *B. subtilis* strain lysogenized with the Phi-3-T, hybridizes with the Phi-3-T cRNA. Specificity of staining is demonstrated by the absence of hybridization with the pSC101 cRNA. Interestingly, the SB168 strain, sensitive to Phi-3-T, also hybridizes to the phage cRNA. SB591, a thymine requiring derivative of SB168, does

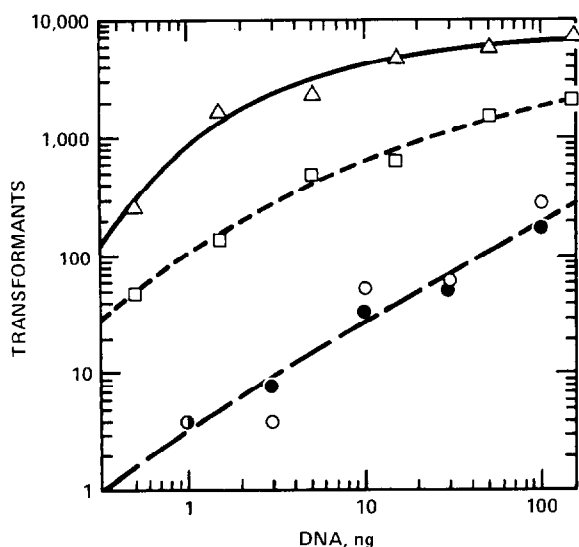


FIG. 3. Transformation of *B. subtilis* to thymine independence with Phi-3-T (triangles) and pFT24 (open circles) DNAs, as well as with *Bam*-cleaved Phi-3-T (squares) and FT24 (closed circles) DNAs.



FIG. 4. Hybridization to *B. subtilis* colonies. Bacteria were streaked on nitrocellulose filter disks, and prepared for *in situ* hybridization. The filter was then cut; left half was hybridized with Phi-3-T, right with pSC101 <sup>32</sup>P-labeled cRNA (about 5 × 10<sup>5</sup> cpm). (A) SB168, (B) SB168(Phi-3-T), (C) SB591*thy*<sup>-</sup>, (D) SB591 transformed with Phi-3-T DNA to *thy*<sup>+</sup>, (E) SB591 transformed with pFT23 DNA, (F) SB591 transformed with pFT24 DNA, (G) *E. coli* C600(pSC101).

not hybridize with the Phi-3-T cRNA: the mutagenesis has apparently deleted sequences complementary to the phage. However, this strain, transformed with either the Phi-3-T or the hybrid plasmid DNAs, does hybridize with Phi-3-T cRNA. The low extent of hybridization displayed by clones of SB591 transformed with the Phi-3-T DNA, as well as their sensitivity to Phi-3-T phage, indicates that only the limited amount of the phage genome has been inserted in the chromosome.

Additional evidence for the presence of Phi-3-T sequences in the hybrid plasmids has been obtained by using the hybridization technique developed by Southern (12). (i) Phi-3-T cRNA binds to all the segments obtained from hybrid plasmids by *Eco*RI cleavage, except the one corresponding to pSC101 part of the molecule. (ii) cRNAs synthesized on each of the hybrid plasmid templates (including pFT33) hybridize to segments 5a, 18, and 23 of the phage DNA [except for pFT24 cRNA which does not hybridize to the latter two: this is expected since pFT24 does not carry these segments (Fig. 1)].

Additional evidence was obtained by the electron microscopic inspection of the heteroduplex molecules formed between the phage and the plasmid DNAs. A double-stranded region was found in all cases. Its length was exactly that measured by gel electrophoresis of the new segment of pFT23 and 24. For pFT25, the insert length corresponded to that length augmented by a molecular weight of 0.9 million (length of region D, Fig. 1) which indicates a Phi-3-T origin of that region. pFT33 was not analyzed.

#### Repetition within the Phi-3-T genome

All pFT cRNAs hybridize to DNA bands 3, 6, 7, 10, 12, and 14 of the Phi-3-T restriction pattern in addition to bands already discussed above (not shown). This could stem from redundant regions within the phage genome. Incomplete degradation

seems to be ruled out as an explanation, because the 10-fold increase in either the amount of *EcoRI* or the time of incubation did not change the described pattern. Similarly, the genetic heterogeneity of plasmids cannot explain the observed hybridization because the cleaved plasmid DNAs appear to hybridize both their own and the Phi-3-T cRNA exclusively to segments displayed in Fig. 1.

Supporting evidence for the repetition within the phage genome comes from the fact that a segment with a molecular weight of 2.1 million that coelectrophoreses with the phage segment 10, complements the thymine deficiency of *E. coli*, when cloned in the plasmid pMB9 (this hybrid plasmid was denoted pFT501). pFT501 hybrid DNA transforms *B. subtilis* at an efficiency similar to that observed with other pFTs (Table 2). The insert is homologous with the part of the segment A of the other four pFTs (Fig. 1) as revealed by heteroduplex analysis. RNA complementary to pFT501 DNA hybridizes to segments 5, 6, 10, 12, and 14 of the phage.

An alternative method to analyze repetitions, the heteroduplex analysis, revealed only one region of homology between Phi-3-T and pFT24, at a distance about 48% from one of the phage DNA. That could be explained by the intrinsic difficulty in analyzing heteroduplexes of such a size, so that a tandem duplication of homologous region might have been undetected.

### Promoter of the *thy* gene in hybrid plasmids

The *thy* gene appears to be under control of a Phi-3-T promoter within segment A (Fig. 1) rather than the promoter within the vector DNA, because: (i) the segment is oriented in the opposite directions in different pFTs (Fig. 1); nevertheless, all the plasmids display the same *thy*<sup>+</sup> character; (ii) the insert cloned in the ColEI-amp plasmid (3) retains the biological activity regardless of its orientation.

## DISCUSSION

*In vitro* insertion of foreign sequences into various replicons creates so-called molecular chimeras. One can reasonably expect that some of those will be better adopted to the intracellular environment than the others. At least two reasons might be invoked. The insert might interfere with the maintenance of the plasmid itself (its replication, for example) or exert some harmful effects on the cell (possibly due to the expression of its genetic information). It would not be surprising then to find examples of evolution of hybrid plasmids from a less to a better adopted form, possibly *via* deletion of harmful sequences. Two of the hybrid plasmids created in course of this work (pFT25 and 33) seem to have undergone such a rearrangement, which has resulted in replacement of the part of pSC101 with the Phi-3-T sequences. We are not aware of any extended homology between the two genomes parental to the hybrid plasmids, which could be invoked in order to interpret the recombination event leading to the observed structures.

One of our hybrid plasmids (pFT33) has acquired an additional segment ("E", Fig. 1) which has a structure typical of transposons (the best studied examples are Tc<sup>r</sup> and Ap<sup>r</sup> genetic elements (16–18). Its spontaneous appearance seem to testify to the abundance of such structures in bacteria.

*EcoRI* cleavage of the Phi-3-T DNA does not occur within the *thy* gene(s), because the segments 5a and 10 of the Phi-3-T complement thymine deficiency in *E. coli* environment, and can transform *thy*<sup>−</sup> *B. subtilis* to prototrophy. However, transforming activity of the phage DNA is 1000-fold impaired upon cleavage. It is, therefore, likely that the decrease in bio-

logical activity is due to the decrease in size upon cleavage of the DNA carrying the *thy* gene. We have observed a similar phenomenon for chromosomal markers of *B. subtilis* (6).

Phi-3-T sequences were detected by hybridization in each of about 100 *B. subtilis* clones transformed to thymine independence with the hybrid plasmid DNAs while the pSC101 sequences could not be revealed. Our attempts to transform *B. subtilis* to Tc<sup>r</sup> by using the hybrid DNA, and by selecting either directly for the resistance, or by replica-plating of *thy*<sup>+</sup> transformants, have consistently failed. It seems, therefore, likely that a mechanism of excision operates on the hybrid plasmid DNAs during transformation of *B. subtilis* cells.

DNA inserts cloned in pSC101 are homologous along their entire length to the Phi-3-T DNA (with the exception of pFT33, see above). Because the hybrid plasmids confer the *thy*<sup>+</sup> character on *E. coli*, the *thy* gene they embody is the gene of Phi-3-T origin. Therefore, a gene which functions in a Gram-positive organism (*B. subtilis*) can also function in a Gram-negative one (*E. coli*). One similar example has been previously reported: the ampicillin gene of the *S. aureus* plasmid conferred resistance to *E. coli* when cloned in the pSC101 plasmid (19). The *thy* gene can be expressed extrachromosomally in two distinct bacterial species, but furthermore it is subject to integration into the *B. subtilis* chromosome wherein it functions as a typical chromosomal gene. This result tends to bolster the prediction that many genes will remain functional when exchanged between two prokaryotic species. This notion is supported by the finding that two other *B. subtilis* chromosomal genes, *leu* and *ura*, have been demonstrated to function in *E. coli* (N. Y. Chi, in preparation). In addition, our evidence indicates that it is very probable that the *thy* gene uses its own promoter in *E. coli*: this opens new approaches to an experimental assessment of the universality of genetic control.

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